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(54) Title: IMPROVEMENTS IN AND RELATING TO A METHOD OF DNA TESTING FOR _____ MYCOBACTERIUM PARATUBERCULOSIS STRAINS

(57) Abstract: The present invention relates to the discovery of a DNA sequence in sheep types of *M. paratuberculosis* that differs from the homologous sequence in cattle types of *M. paratuberculosis*. The invention also provides a nucleic acid amplification technique based on these differences that can be used to distinguish strains of the cattle type from strains of both the sheep types of *M. paratuberculosis*. The invention also relates to use of these sequences in a nucleic acid amplification technique to distinguish all strains of *M. paratuberculosis* from other strains of the MAI complex and from strains of the *M. tuberculosis* complex.

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IMPROVEMENTS IN AND RELATING TO A METHOD OF DNA TESTING FOR MYCOBACTERIUM PARATUBERCULOSIS STRAINS

TECHNICAL FIELD

This invention relates to improvements in and relating to a method of DNA testing. In particular this invention relates to nucleic acid sequences of
5 *Mycobacterium paratuberculosis* and their use in a method for identifying different strains of *M. paratuberculosis* and distinguishing strains of *M. paratuberculosis* from other mycobacterial species. This invention also provides an aid in the diagnosis of diseases caused by Mycobacterial species in human and animal medical practice.

10 BACKGROUND ART

Mycobacteria are rod-shaped, acid-fast, aerobic bacilli that do not form spores. A moderate number of slow-growing mycobacterial species are major pathogens for humans and/or animals. For example, paratuberculosis is a very widespread animal health problem which causes major economic losses in farming of
15 ruminant animals particularly in the dairy industry. The development of robust diagnostic tests to distinguish different mycobacterial species and to characterise subspecies, groups and types of related strains within a species is of prime importance.

Paratuberculosis or Johne's disease is a chronic granulomatous enteritis that can
20 affect all domestic and wild ruminants causing reduced food intake, weight loss and death. The disease is present in most countries and results in significant production losses. The causative organism, *Mycobacterium avium* subsp. *paratuberculosis* (basonym *M. paratuberculosis*) (Harris *et al.*, 2001) has also been implicated as the etiologic agent of Crohn's disease in humans and is a
25 member of the MAI complex, a group of closely related species which includes

Mycobacterium intracellulare and all subspecies of *M. avium*. For taxonomic purposes, *M. avium* is divided into the three subspecies *M. avium* subsp. *avium* (although in most publications this subspecies is still referred to as *M. avium*), *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *silvaticum* (Thorel *et al.*, 5 1990). While *M. paratuberculosis* appears to be an obligate pathogen, closely-related organisms of the MAI complex that share many common antigens with *M. paratuberculosis* are widespread throughout the environment. Exposure of animals to these environmental organisms is probably responsible for the lack of sensitivity and specificity of antigen-based diagnostic tests for *M.* 10 *paratuberculosis*. Other problems that have made this disease particularly difficult to control are the very slow growth of the organism on artificial culture, and the ability of the organism to survive in many animals for years without causing any overt disease (Chiodini *et al.*, 1984; Harris and Barletta, 2001).

Two recent discoveries have shown that the spread of *M. paratuberculosis* may be 15 more complicated than previously believed and emphasise the need for the development of new diagnostic tools. First, the organism has been reported to survive normal milk pasteurisation (Grant *et al.*, 2002). Since pasteurised milk is widely consumed in many countries, this survival provides a route by which large sections of the population can be exposed to this obligate pathogen and supports 20 the case of those who claim that it causes Crohn's disease in humans (Hermon-Taylor *et al.*, 2000; Harris and Barletta, 2001). Second, *M. paratuberculosis* has also been isolated in the United Kingdom from common wild non-ruminant animals such as rabbits, foxes, stoats and crows (Beard *et al.*, 2001). This finding complicates epidemiological studies, as previously it had been believed that 25 spread of the disease occurred only from ruminants, either directly from one animal to another or through infected milk or by grazing on pasture infected by organisms shed from another infected ruminant (Chiodini *et al.*, 1984).

The first significant molecular biological development in the study of *M. paratuberculosis* was the discovery of multiple copies of an insertion sequence IS900 (Collins *et al.*, 1989; Green *et al.*, 1989). This sequence has been found to be specific for *M. paratuberculosis* and is now widely used as the basis for diagnostic tests that use DNA amplification (Collins *et al.*, 1993; Fang *et al.*, 2002). Related insertion sequences have been found in other members of the MAI complex (Kunze *et al.*, 1991; Englund *et al.*, 2002) and the finding that the most recently discovered sequence is 94% identical to IS900 has raised doubts about the specificity of tests based on parts of the IS900 sequence (Englund *et al.*, 2002).

There would be advantages in having a range of sequences that have a high probability of being specific to *M. paratuberculosis* so that new tests could be widely trialled to determine which sequences are truly unique to this species. Sequencing of the genomes of both an *M. paratuberculosis* and an *M. avium* subsp. *avium* strain is currently in progress and a range of sequences that might differ between these two strains have been identified (Bannantine, 2002). Whether all these differences are real cannot be determined until the sequencing of both genomes is completed but even then the genetic diversity of different MAI strains is such (Falkinham, 1999) that it will be some years before the degree of specificity of these sequences can be determined for a wide range of strains in the different subspecies.

Isolates of *M. paratuberculosis* were first characterised into cattle and sheep types in 1990 (Collins *et al.*, 1990) on the basis of restriction fragment length polymorphisms (RFLPs) of the insertion sequence IS900 and this largely correlates with the difficulty of primary isolation of sheep types (Collins *et al.*, 1990, Pavlik *et al.*, 1999). The distinction into cattle and sheep types is epidemiologically useful, as cattle and sheep are preferentially infected with their named types while other ruminant species such as deer and goats appear to be

infected more easily with either type (Collins *et al.*, 1990; de Lisle *et al.*, 1993; Pavlik *et al.*, 1999; Whittington *et al.*, 2000). Sheep strains from Canada (Collins *et al.*, 1990) and subsequently from South Africa (de Lisle *et al.*, 1992) and Iceland (de Lisle *et al.*, 1993) were found to have RFLP patterns that clustered in a group that was different from that of cattle types and other sheep types and were classified as belonging to a third or intermediate type. A careful comparison of members of these three RFLP types revealed that the pattern of the intermediate type was more closely related to patterns of the other sheep type than to patterns of the cattle type (Pavlik *et al.*, 1999) and for this reason, and also because of its epidemiological association with sheep, this intermediate type is better referred to as a variant or second sheep type.

At present, DNA amplification testing for paratuberculosis where both cattle and sheep types are potentially present, involves a PCR assay based on IS900 to confirm the presence of *M. paratuberculosis* followed by a PCR based on IS1311 whose product is then subjected to restriction endonuclease analysis (Whittington *et al.*, 2000). This two-step PCR analysis approach is performed because IS1311 is not unique for *M. paratuberculosis* and is also found in *M. avium* subsp. *avium* (Collins *et al.*, 1997), but some copies of IS1311 in *M. paratuberculosis* have polymorphisms that are specific for the cattle and sheep types and the polymorphisms can be detected by digesting the IS1311 PCR product with appropriate restriction enzymes (Marsh *et al.*, 1999).

Thus, it would be useful if there could be provided a single PCR diagnostic test which can distinguish between *M. paratuberculosis* and other mycobacterial species of the MAI complex and also within the same test distinguish between sheep and cattle type strains of *M. paratuberculosis*.

All references, including any patents or patent applications cited in this

specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents form part of the common general knowledge in the art, in New Zealand or in any other country.

It is acknowledged that the term 'comprise' may, under varying jurisdictions, be attributed with either an exclusive or an inclusive meaning. For the purpose of this specification, and unless otherwise noted, the term 'comprise' shall have an inclusive meaning - i.e. that it will be taken to mean an inclusion of not only the listed components it directly references, but also other non-specified components or elements. This rationale will also be used when the term 'comprised' or 'comprising' is used in relation to one or more steps in a method or process.

It is an object of the present invention to address the foregoing problems or at least to provide the public with a useful choice.

Further aspects and advantages of the present invention will become apparent from the ensuing description which is given by way of example only.

SUMMARY OF INVENTION

The present invention relates to the discovery of a DNA sequence in sheep types of *M. paratuberculosis* that differs from the homologous sequence in cattle types of *M. paratuberculosis*. The invention also provides a nucleic acid amplification technique based on these differences that can be used to distinguish strains of the cattle type from strains of both the sheep types of *M. paratuberculosis*. The invention also relates to use of these sequences in a nucleic acid amplification

technique to distinguish all strains of *M. paratuberculosis* from other strains of the MAI complex and from strains of the *M. tuberculosis* complex.

DISCLOSURE OF INVENTION

According to a first aspect of the present invention there is provided a nucleic acid molecule of a sheep type of *M. paratuberculosis* said molecule comprising SEQ ID NO. 1 or a complement thereof.

According to a second aspect of the present invention there is provided a probe comprising SEQ ID NO.1 or a complement thereof.

According to a third aspect of the present invention there is provided a probe comprising at least 6 contiguous nucleotides selected from nucleotides 1 – 35 of SEQ ID NO. 1 or a complement thereof.

Preferably, the probe substantially as described above may include at least 10-12 contiguous nucleotides selected from nucleotides 1 – 35 of SEQ ID NO. 1 or a complement thereof.

More preferably the probe substantially as described above may include more than 20 contiguous nucleotides selected from nucleotides 1 – 35 of SEQ ID NO. 1 or a complement thereof.

According to a fourth aspect of the present invention there is provided a probe comprising at least 6 contiguous nucleotides selected from nucleotides 230 – 260 of SEQ ID NO. 1 or a complement thereof.

Preferably the probe substantially as described above may include 10-12 contiguous nucleotides selected from nucleotides 230 – 260 of SEQ ID NO. 1 or a complement thereof.

More preferably the probe substantially as described above may include more than 20 contiguous nucleotides selected from nucleotides 230 – 260 of SEQ ID NO. 1 or a complement thereof.

5 According to a fifth aspect of the present invention there is provided a use of a nucleic acid molecule or probe substantially as described above for detecting the presence of sheep types of *M. paratuberculosis*.

According to a sixth aspect of the present invention there is provided a use of SEQ ID NO 2 or, a fragment or a complement, thereof for detecting the presence of cattle types of *M. paratuberculosis*.

10 According to a seventh aspect of the present invention there is provided a method of distinguishing between cattle and sheep types of *M. paratuberculosis* comprising the step of comparing differences between the nucleotide sequences of SEQ ID NO. 1 and SEQ ID NO. 2 or complements of said sequences.

15 According to a eighth aspect of the present invention there is provided a method of detecting the presence of *M. paratuberculosis* in a sample via a nucleic acid amplification technique said method comprising the steps of:

- a) taking a sample from an animal or any other source;
- b) extracting nucleic acids from the sample or culturing mycobacteria from the sample and extracting nucleic acids from the mycobacterial culture;
- 20 c) performing a nucleic acid amplification technique; and
- d) determining the identity of the amplification product.

Preferably animals may include cattle, sheep, deer, goats, ferrets, rabbits and humans.

According to a ninth aspect of the present invention there is provided a method substantially as described above wherein step d) of the method comprises identifying the presence of at least 6 nucleotides of the nucleic acid molecule comprising SEQ ID NO. 1 or a complement thereof.

- 5 According to a tenth aspect of the present invention there is provided a method substantially as described above wherein step d) of the method comprises identifying the presence of 10-12 contiguous nucleotides of the nucleic acid molecule comprising SEQ ID NO. 1 or a complement thereof.

- 10 According to an eleventh aspect of the present invention there is provided a method substantially as described above wherein step d) of the method comprises identifying the presence of at least 15 contiguous nucleotides of the nucleic acid molecule comprising SEQ ID NO. 1 or a complement thereof.

- 15 According to a twelfth aspect of the present invention there is provided a method substantially as described above wherein step d) of the method comprises identifying the presence of substantially 20 contiguous nucleotides of the nucleic acid molecule comprising SEQ ID NO. 1 or a complement thereof.

- 20 According to a thirteenth aspect of the present invention there is provided a method substantially as described above wherein step c) utilizes one oligonucleotide primer complementary to at least 6 contiguous nucleotides of SEQ ID NO. 1 or a complement thereof; and one oligonucleotide primer complementary to at least 6 nucleotides of IS900 or a complement thereof.

According to a fourteenth aspect of the present invention there is provided a method substantially as described above wherein step c) utilizes one oligonucleotide primer complementary to 10-12 contiguous nucleotides of SEQ

ID NO. 1 or a complement thereof and one oligonucleotide primer complementary to 10-12 nucleotides of IS900 or a complement thereof.

According to a fifteenth aspect of the present invention there is provided a method substantially as described above wherein step c) utilizes one oligonucleotide
5 primer complementary to substantially 15 contiguous nucleotides of SEQ ID NO. 1 or a complement thereof and one oligonucleotide primer complementary to substantially 15 nucleotides of IS900 or a complement thereof.

According to a sixteenth aspect of the present invention there is provided a method substantially as described above wherein step c) utilizes one
10 oligonucleotide primer complementary to substantially 20 contiguous nucleotides of SEQ ID NO. 1 or a complement thereof; and one oligonucleotide primer complementary to substantially 20 nucleotides of IS900 or a complement thereof.

According to a seventeenth aspect of the present invention there is provided a method substantially as described above wherein step c) of the method comprises
15 identifying the presence of at least 6 contiguous nucleotides of the nucleic acid molecule comprising SEQ ID NO. 2 or a complement thereof.

According to an eighteenth aspect of the present invention there is provided a method substantially as described above wherein step d) of the method comprises identifying the presence of 10-12 contiguous nucleotides of the nucleic acid
20 molecule comprising SEQ ID NO. 2 or a complement thereof.

According to a nineteenth aspect of the present invention there is provided a method substantially as described above wherein step d) of the method comprises identifying the presence of at least 15 contiguous nucleotides of the nucleic acid molecule comprising SEQ ID NO. 2 or a complement thereof.

According to a twentieth aspect of the present invention there is provided a method substantially as described above wherein step d) of the method comprises identifying the presence of approximately 20 contiguous nucleotides of the nucleic acid molecule comprising SEQ ID NO. 2 or a complement thereof.

- 5 According to a twenty-first aspect of the present invention there is provided a method substantially as described above wherein step c) utilizes one oligonucleotide primer complementary to at least 6 contiguous nucleotides of SEQ ID NO. 2 or a complement thereof; and one oligonucleotide primer complementary to at least 6 nucleotides of IS900 or a complement thereof.
- 10 According to a twenty-second aspect of the present invention there is provided a method substantially as described above wherein step c) utilizes one oligonucleotide primer complementary to 10-12 contiguous nucleotides of SEQ ID NO. 2 or a complement thereof; and one oligonucleotide primer complementary to 10-12 nucleotides of IS900 or a complement thereof.
- 15 According to a twenty-third aspect of the present invention there is provided a method substantially as described above wherein step c) utilizes one oligonucleotide primer complementary to substantially 15 contiguous nucleotides of SEQ ID NO. 2 or a complement thereof; and one oligonucleotide primer complementary to substantially 15 nucleotides of IS900 or a complement thereof.
- 20 According to a twenty-fourth aspect of the present invention there is provided a method substantially as described above wherein step c) utilizes one oligonucleotide primer complementary to substantially 20 contiguous nucleotides of SEQ ID NO. 2 or a complement thereof; and one oligonucleotide primer complementary to substantially 20 nucleotides of IS900 or a complement thereof.

According to a twenty-fifth aspect of the present invention there is provided a use of a probe comprising at least 6 contiguous nucleotides selected from the nucleic acid comprising SEQ ID NO. 2 or a complement thereof.

5 According to a twenty-six aspect of the present invention there is provided a use of a probe comprising substantially 10-12 contiguous nucleotides selected from the nucleic acid comprising SEQ ID NO. 2 or a complement thereof.

According to a twenty-seventh aspect of the present invention there is provided a use of a probe comprising at least 15 contiguous nucleotides selected from the nucleic acid comprising SEQ ID NO. 2 or a complement thereof.

10 According to a twenty-eighth aspect of the present invention there is provided a use of a probe comprising at least 20 contiguous nucleotides selected from the nucleic acid comprising SEQ ID NO. 2 or a complement thereof.

According to a twenty-ninth aspect of the present invention there is provided use of SEQ ID NO.1 and/or SEQ ID NO. 2, or a fragment or complement thereof,
15 substantially as described above to determine whether a strain of either a sheep type or a cattle type of *M. paratuberculosis* is present in a sample.

According to a thirtieth aspect of the present invention there is provided a use of SEQ ID NO.1, or a fragment or complement thereof, to distinguish any strain of *M. paratuberculosis* from any other strain of the MAI complex which may be
20 present in a sample.

According to a thirty-first aspect of the present invention there is provided a use of SEQ ID NO.2, or a fragment or complement thereof, above to distinguish any strain of *M. paratuberculosis* from any other strain of the MAI complex which may be present in a sample.

According to a thirty-second aspect of the present invention there is provided a use of SEQ ID NO.1, or a fragment or complement thereof to distinguish any strain of *M. paratuberculosis* from any strain of the *M. tuberculosis* complex which may be present in a sample.

- 5 According to a thirty-third aspect of the present invention there is provided a use of SEQ ID NO.2, or a fragment or complement thereof, to distinguish any strain of *M. paratuberculosis* from any strain of the *M. tuberculosis* complex which may be present in a sample.

- 10 According to a thirty-fourth aspect of the present invention there is provided a use of SEQ ID NO. 1, or a fragment or complement thereof to detect the presence of *M. paratuberculosis* as a causative agent of Johne's disease or Crohn's disease.

According to a thirty-fifth aspect of the present invention there is provided a use of SEQ ID NO. 2, or a fragment or complement thereof, to detect the presence of *M. paratuberculosis* as a causative agent of Johne's disease or Crohn's disease.

- 15 It will be appreciated by those skilled in the art such that know and use of the nucleotide sequences of SEQ ID NO. 1 and SEQ ID NO. 2 will be useful as an aid in the diagnosis of these diseases.

- 20 The term "sheep type of *M. paratuberculosis*" as used herein refers to a strain of *M. paratuberculosis* which preferentially infects sheep but also may infect other species for example deer, goats and humans but does not preferentially infect cattle.

- 25 The term "cattle type of *M. paratuberculosis*" as used herein refers to a strain of *M. paratuberculosis* which preferentially infects cattle but also may infect other species for example deer, goats and humans but does not preferentially infect sheep.

The term "IS900" as used herein refers to a known DNA sequence that is characteristically present in strains of *M. paratuberculosis* and which is currently used to detect *M. paratuberculosis* species.

5 "Probes" are single-stranded nucleic acid molecules with known nucleotide sequences which are labelled in some way (for example, radioactively, fluorescently or immunologically), which are used to find and mark a target DNA or RNA sequence by hybridizing to it.

10 "Primers" are short nucleic acids, preferably DNA oligonucleotides 15 nucleotides or more in length, which are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand; they can then be extended along the target DNA strand by a polymerase, preferably a DNA polymerase. Primer pairs can be used for amplification of a nucleic acid sequence, e.g. by the polymerase chain reaction (PCR) or other nucleic acid amplification methods well known in the art. PCR-
15 primer pairs can be derived from the sequence of a nucleic acid according to the present invention, for example, by using computer programs intended for that purpose such as Primer (Version 0.5[©] 1991, Whitehead Institute for Biomedical Research, Cambridge, MA).

20 A fragment of a nucleic acid is a portion of the nucleic acid that is less than full length and comprises at least a minimum sequence capable of hybridising specifically with a nucleic acid molecule according to the present invention (or a sequence complementary thereto) such that the fragment can have at least one of the utilities of the nucleic acid of the present invention.

The term "complement" refers to a second single stranded nucleic acid molecule
25 having a nucleotide sequence corresponding to the nucleotide sequence of a first nucleic acid molecule: as determined by the base pairing of adenosine to Thymine

and of guanine to cytosine as occurs in a double stranded DNA molecule.

The term "nucleic acid amplification technique" as used herein may generally be considered to refer to the polymerase chain reaction or PCR. However, it may equally refer to other equivalent techniques for amplifying nucleic acids known to those skilled in the art.

The term "polymerase chain reaction or PCR" as used herein refers to a system for *in vitro* amplification of DNA. Two synthetic oligonucleotide primers, which are complementary to two regions of the target DNA (one for each strand) to be amplified, are added to the target DNA (that need not be pure), in the presence of excess deoxynucleotides and Taq polymerase, a heat-stable DNA polymerase. In a series of temperature cycles, the target DNA is repeatedly denatured, annealed to the primers (typically at 50-60°C) and a daughter strand extended from the primers. As the daughter strands themselves act as templates for subsequent cycles, DNA fragments matching both primers are amplified exponentially.

The nucleic acid molecule may be an RNA, cRNA, genomic DNA or cDNA molecule, and may be single- or doublestranded. The nucleic acid molecule may also optionally comprise one or more synthetic, non-natural or altered nucleotide bases, or combinations thereof. The detection of the amplified nucleic acid may be by any of a wide range of techniques known to those skilled in the art, including but not limited to size separation techniques such as gel electrophoresis, probe detection systems either on solid supports or in solution and DNA microarray techniques.

BRIEF DESCRIPTION OF DRAWINGS

Further aspects of the present invention will become apparent from the following description which is given by way of example only and with reference to the accompanying drawings in which:

- 5 Figure 1 Shows nucleic acid SEQ ID NO. 1;
- Figure 2 Shows nucleic acid SEQ ID NO 2;
- Figure 3 Shows PCR products from DNA of two sheep types and two cattle
 types of *M. paratuberculosis* performed with oligonucleotide
 primers DMC136 and DMC137 at an annealing temperature of
10 50°C;
- Figure 4 Shows Alignment of homologous DNA sequences from cattle and
 sheep types of *M. paratuberculosis*. Identical nucleotides in both
 sequences are shaded, arrows indicate the identity and direction of
 the oligonucleotide primers used, the tandem DNA sequence
15 present in the sheep type is shown in boxes, the pallindromic region
 is underlined, the complement of the 5' end of the coding sequence
 of the putative gene involved in phage attachment is shown in
 lower case text, and dots indicate no sequence;
- Figure 5 Shows Diagrammatic representation, relative to the chromosome of
20 the cattle type, of the position of SEQ ID NO. 1 and the points of
 insertion of the tandem repeat and the likely copy of IS900 in the
 sheep type; and
- Figure 6 PCR products from cattle and sheep types of *M. paratuberculosis*
 amplified with the three oligonucleotide primers DMC529,

DMC531, and DMC533. Lanes: 1 and 11, molecular size markers; 2-5, cattle types; 6-9, sheep types; 10, negative control.

Figure 7 PCR products from cattle types of *M. paratuberculosis* amplified with the three oligonucleotide primers DMC529, DMC531, and DMC533 from BACTEC 12B radiometric medium containing egg yolk. Lanes: 1-3, BACTEC cultures of *M. paratuberculosis*; 4, positive control DNA from cattle type of *M. paratuberculosis*; 5, negative control; 7 molecular size markers.

BEST MODES FOR CARRYING OUT THE INVENTION

10 *Experimental*

Non-limiting examples illustrating the invention will now be provided. It will be appreciated that the above description is provided by way of example only and variations in the materials and technique used which are known to those skilled in the art are contemplated.

15 The present invention provides a DNA sequence, SEQ ID NO. 1 (Fig. 1), that is unique to sheep types of *M. paratuberculosis* and provides the use of SEQ ID NO. 2 (Fig. 2) for diagnostic testing for organisms of the MAI complex. The present invention also provides for the specific use of SEQ ID NO. 1 and SEQ ID NO. 2 to distinguish between sheep types and cattle types of *M. paratuberculosis* and to
20 distinguish all *M. paratuberculosis* strains from other strains of the closely related MAI complex and from strains of the *M. tuberculosis* complex. A PCR diagnostic test using three oligonucleotide primers is given as an example of the utility of the invention but the invention is not limited to these oligonucleotides or to the use of PCR and a wide range of other diagnostic tests based on these sequences, their
25 complements or any RNA or protein that they specify is envisaged.

Discovery of SEQ ID NO. 1

The experimental work described here is also summarised in Collins *et al.*, (2002). The strains of the MAI complex used in this work are given in Table 1. Oligonucleotide primers used in the work are given in Table 2. Strains were

5 cultured using specialised mycobacterial media (Collins *et al.*, 1997; Whittington *et al.*, 1999). Purified DNA was extracted as described previously (Collins *et al.*, 1990). When DNA from strains of both the sheep and cattle types of *M. paratuberculosis* was subjected to PCR at an annealing temperature of 50°C using primers DMC136 and DMC137 directed outwards from each end of IS900, only

10 DNA from sheep types gave a major product between 300 bp and 400 bp (Fig. 3). Subsequently, it was observed that the same 342 bp product was obtained if only one PCR primer (DMC136) was used. The PCR product was extracted from the gel, re-amplified at an annealing temperature of 65°C, cloned into pBluescript KSII (Stratagene) and sequenced (Fig. 1). Comparison of this sequence using the

15 programme BLAST against the partially completed sequence of the genome of a cattle type of *M. paratuberculosis* (National Centre for Biotechnology Information database [<http://www.ncbi.nlm.nih.gov/>]) indicated a high degree of homology to positions 1020 – 1316 of SEQ ID NO. 2 (Fig. 2), denoted as contig 249 in the database. Comparisons using the GAP programme of GCG (Wisconsin Package

20 Version 10.2, Genetics Computer Group, Madison, Wisconsin) delineated two major differences (Fig. 4). First, the sheep type but not the cattle type has a tandem repeat of a 12 bp sequence followed by a 4 bp linker that together contain a 14 bp pallindromic sequence. Second, the cattle type was not homologous to DMC136 at the 5' end. This indicated that an IS900 element was inserted at the 5' end of SEQ

25 ID NO. 1 in the sheep type but not in the homologous region of SEQ ID NO. 2 in the cattle type. Further investigation using DMC505 and DMC507 indicated that both types have similar sequences at the 3' end because these primers worked

equally well in a PCR reaction with an annealing temperature of 60°C on DNA from both sheep and cattle types and gave products differing by only 16 bp. It thus appears that the product shown in Fig. 3 was produced by using the single primer DMC136 because this primer is completely homologous to DNA of the ovine type at the 5' end of SEQ ID NO. 1 (Figs. 1 and 4) but homologous to only about the last 10 nucleotides at the 3' end. For this reason, the final nine nucleotides of SEQ ID NO. 1 (Fig. 1) are shown in lower case text. The most likely explanation for homology of the 5' end of SEQ ID NO. 1 to DMC136 is that a copy of IS900 is inserted at this position in the genome of sheep types but not cattle types and this is shown in diagrammatic form in Fig.5. Confirmation of the presence of this copy of IS900 was provided by performing a PCR on both sheep and cattle types of *M. paratuberculosis* using the oligonucleotide primers DMC137, which reads out of IS900 from the opposite end to DMC136, and DMC531 which would be expected to read towards the DMC137 end of IS900 in sheep types of *M. paratuberculosis*. As expected, this pair of primers gave a product of the expected size with sheep types of *M. paratuberculosis* but no product with cattle types. Clearly, to one with skill in the art this provides alternative regions of IS900 and SEQ ID NO. 2 to those used in the PCR example below and these alternative regions could be used for designing oligonucleotide primers and constructing PCR tests that potentially have similar utility to that of the example. Further analysis of SEQ ID NO. 1 and SEQ ID NO 2 using other GCG programmes showed that both the tandem repeat and the difference between sheep and cattle types at the 5' end of SEQ ID NO. 1 are in or adjacent to likely coding sequences one of which has high homology to a gene whose product is involved in phage attachment (Barsom and Hatfull, 1996). These differences may therefore be important in determining the host preference of sheep and cattle types. If this is the case, these DNA differences observed between cattle and sheep types may be a very widespread or even ubiquitous phenomenon. Comparison of SEQ ID NO. 1 and SEQ ID NO 2 (Figs. 1 and 2) to

the incomplete genome sequence of an *M. avium* subsp. *avium* strain (National Centre for Biotechnology Information database [<http://www.ncbi.nlm.nih.gov/>]) did not identify any closely homologous sequences apart from the first 285 bp of SEQ ID NO 2. This indicated that SEQ ID NO. 1 and most of SEQ ID NO. 2 might not be present in *M. avium* subsp. *avium* and that these sequences could thus be used for constructing tests to distinguish between *M. paratuberculosis* and *M. avium* subsp. *avium*.

Development of a PCR assay

A PCR assay was developed using a GeneAmp PCR System 9600 (Applied Biosystems) and the three primers DMC529, DMC531, DMC533 (Table 2 and Fig. 4) under the following conditions: 1 cycle at 95°C, 3 min; 25 cycles at 60°C, 30 s, 72°C, 30 s, 94°C, 30 s; 1 cycle at 72°C, 7min. DNA from all 19 strains of the cattle type (Table 1) gave the expected product of 310 bp, while DNA from all 12 strains of the sheep type (Table 1) gave the expected product of 162 bp (Fig. 6). A PCR product was not observed for any of the wide range of strains of the MAI complex (Table 1) that did not contain IS900 and were not *M. paratuberculosis*. No PCR product was observed with strains of *Mycobacterium tuberculosis* and *Mycobacterium bovis* (Table 1) which are members of the *M. tuberculosis* complex. This group of organisms which cause tuberculosis in mammals comprise the following species: *M. tuberculosis*, *M. bovis*, *Mycobacterium bovis* subsp. *caprae*, *Mycobacterium microti* and *Mycobacterium canettii*. *M. tuberculosis* causes most of the tuberculosis in humans and *M. bovis* causes tuberculosis in a wide range of mammals including humans, cattle and deer. In some situations there is diagnostic utility in having a fast diagnostic test such as PCR to distinguish between samples from animals infected with strains of the *M. tuberculosis* complex and those infected with *M. paratuberculosis* (de Lisle *et al.*, 1993).

The MAI complex covers a relatively broad group of genetically related mycobacteria that, with the exception of *M. paratuberculosis*, are found in many environmental niches and are occasional mammalian pathogens. Because of the potential of these organisms to confuse the diagnosis of paratuberculosis, strains of the MAI complex tested in this study were weighted towards those that had been isolated from humans or from a range of different animal hosts and that might be expected to be most closely related to *M. paratuberculosis* (Collins *et al.*, 1997). The fact that none of these strains was positive in the PCR assay gives a high level of confidence in the utility of the test to distinguish between strains of *M. paratuberculosis* and other strains of the MAI complex. In the case of *M. paratuberculosis*, the inclusion of 10 strains from five other countries, including sheep strains from Canada, South Africa and Iceland, enabled a cross-section of strains with different IS900 RFLP types to be tested. In all cases, the PCR results were consistent with this RFLP division into sheep and cattle types, indicating that tests based on SEQ ID NO. 1 and SEQ ID NO. 2 should have wide utility for distinguishing between sheep and cattle types of *M. paratuberculosis* in many countries. Because of the association of *M. paratuberculosis* with Crohn's disease in humans, assays based on SEQ ID NO. 1 and SEQ ID NO. 2 not only have the potential to be widely applicable to epidemiological and other studies of paratuberculosis but will also have utility in the field of Crohn's disease.

Detection of *M. paratuberculosis* in modified BACTEC medium

For detection of *M. paratuberculosis* in modified BACTEC 12B radiometric medium containing egg yolk (Whittington *et al.*, 1999), 200 µl of medium containing the organisms was added to approximately 0.5 ml glass beads (Qbiogene Lysing Matrix B) and extracted using QIAamp DNA Stool Mini Kit (QIAGEN) by treating the sample plus added glass beads as if it was a stool sample and following the manufacturer's protocols with minor modifications as

outlined. The sample plus added glass beads was vortexed in 1 ml proprietary ASL buffer from the QIAamp kit for two periods of 20 sec each in a Ribolyser (ThermoSavant FastPrep Cell Disrupter) set on 6.5 with 1 min cooling on ice between each period. The suspension was heated at 95 °C for 10 min, vortexed for 15 sec and centrifuged. Approximately 800 µl of supernatant was removed, made up to 1.2 ml with proprietary ASL buffer and treated from then on as detailed in the Manufacturer's protocols. Briefly, the 1.2 ml sample was vortexed with an InhibitEX tablet and incubated for 1 min at room temperature. After centrifuging twice, 200 µl of supernatant was added to 15 µl Proteinase K, 200 µl proprietary AL buffer was added and the mixture was vortexed for 15 sec and then incubated at 70 °C for 10 min. The mixture was vortexed with 200 µl absolute ethanol and eluted through a QIAamp (QUIGEN) spin column as detailed in the Manufacturer's protocols. A final eluate of approximately 200 µl was collected and 10 – 20 µl was subjected to PCR with the three primers DMC529, DMC531, DMC533. The PCR conditions used were either 1 cycle at 95°C, 3 min; 42 cycles at 50°C, 30 s, 72°C, 30 s, 94°C, 30 s; 1 cycle at 72°C, 7min; or 1 cycle at 95°C, 3 min; 42 cycles at 45°C, 30 s, 72°C, 30 s, 94°C, 30 s; 1 cycle at 72°C, 7min. PCR products using the last set of PCR conditions above for cattle types of *M. paratuberculosis* cultured in BACTEC medium containing egg yolk are shown in Fig. 7.

Table 1. Strains of the MAI complex and *M. tuberculosis* complex subjected to PCR

No. of strains	With IS900	With IS901	Description	Source
10	0	3	Reference serotypes 1-6, 8-11 of MAI complex*	Dawson **
11	0	7	Cattle, deer and pig isolates of MAI complex*	New Zealand
6	0	5	Bird isolates of MAI complex*	New Zealand
4	0	0	Human isolates of MAI complex*	New Zealand
14	14	—	<i>M. paratuberculosis</i> cattle type	New Zealand
3	3	—	<i>M. paratuberculosis</i> cattle type	Canada
1	1	—	<i>M. paratuberculosis</i> TMC1613; cattle type	USA
1	1	—	<i>M. paratuberculosis</i> 316F; cattle type	UK
7	7	—	<i>M. paratuberculosis</i> sheep type	New Zealand
1	1	—	<i>M. paratuberculosis</i> sheep type	Canada
3	3	—	<i>M. paratuberculosis</i> sheep type	South Africa
1	1	—	<i>M. paratuberculosis</i> sheep type	Iceland
1	1	—	<i>M. tuberculosis</i> H37Rv	USA
2	—	—	<i>M. bovis</i>	New Zealand

* None of these strains of the MAI complex were *M. paratuberculosis*

** See Wards *et al.* (1987)

Table 2. DNA oligonucleotide primers used in this work

Oligonucleotide	Sequence 5' – 3'
DMC136	GCTTGACAACGTCATTGAG
DMC137	CCCTTCAAGAAAGGTAAGG
DMC505	CAAGTTGTCGTA CTCTCGTC
DMC507	TTAGCTGACCTATCTACAGGC
DMC529	TTGACAACGTCATTGAGAATCC
DMC531	TCTTATCGGACTTCTTCTGGC
DMC533	CGGATTGACCTGCGTTTCAC

Aspects of the present invention have been described by way of example only and it should be appreciated that modifications and additions may be made thereto

5 without departing from the scope thereof as defined in the appended claims.

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WHAT WE CLAIM IS:

1. A nucleic acid molecule of a sheep type of *M. paratuberculosis* said molecule comprising SEQ ID NO. 1 or a complement thereof.
2. A probe comprising SEQ ID NO.1 or a complement thereof.
- 5 3. A probe comprising at least 6 contiguous nucleotides selected from nucleotides 1 – 35 of SEQ ID NO. 1 a complement thereof.
4. A probe comprising at least 10-12 contiguous nucleotides selected from nucleotides 1 – 35 of SEQ ID NO. 1 or a complement thereof.
5. A probe comprising at least 20 or more contiguous nucleotides selected
10 from nucleotides 1 – 35 of SEQ ID NO. 1 or a complement thereof.
6. A probe comprising at least 6 contiguous nucleotides selected from nucleotides 230 – 260 of SEQ ID NO. 1 or a complement thereof.
7. A probe comprising at least 10-12 contiguous nucleotides selected from nucleotides 230 – 260 of SEQ ID NO. 1 or a complement thereof.
- 15 8. A probe comprising at least 20 or more contiguous nucleotides selected from nucleotides 230 – 260 of SEQ ID NO. 1 or a complement thereof.
9. The use of a nucleic acid molecule or probe as claimed in any one of claims 1-7 for detecting the presence of sheep types of *M. paratuberculosis*.
- 20 10. The use of SEQ ID NO 2 or, a fragment or complement thereof for detecting the presence of cattle types of *M. paratuberculosis*.

11. A method of distinguishing between cattle and sheep types of *M. paratuberculosis* comprising the step of comparing differences between the nucleotide sequences of SEQ ID NO. 1 and SEQ ID NO. 2 or complements of said sequences.
- 5 12. A method of detecting the presence of *M. paratuberculosis* in a sample via a nucleic acid amplification technique said method comprising the steps of:
 - a) taking a sample from an animal or any other source;
 - b) extracting nucleic acids from the sample or culturing mycobacteria from the sample and extracting nucleic acids from the
10 mycobacterial culture;
 - c) performing a nucleic acid amplification technique; and
 - d) determining the identity of the amplification product.
13. A method as claimed in claim 12 wherein the animals may include cattle, sheep, deer, goats, ferrets, rabbits and humans.
- 15 14. A method as claimed in claim 12 wherein step d) of the method comprises identifying the presence of at least 6 nucleotides of the nucleic acid molecule comprising SEQ ID NO. 1 or a complement thereof.
15. A method as claimed in claim 12 wherein step d) of the method comprises identifying the presence of 10-12 contiguous nucleotides of the nucleic
20 acid molecule comprising SEQ ID NO. 1 or a complement thereof.
16. A method claimed in claim 12 wherein step d) of the method comprises identifying the presence of at least 15 contiguous nucleotides of the nucleic acid molecule comprising SEQ ID NO. 1 or a complement thereof.

17. A method as claimed in claim 12 wherein step d) of the method comprises identifying the presence of substantially 20 contiguous nucleotides of the nucleic acid molecule comprising SEQ ID NO. 1 or a complement thereof.
- 5 18. A method as claimed in claim 12 wherein step c) utilizes one oligonucleotide primer complementary to at least 6 contiguous nucleotides of SEQ ID NO. 1 or a complement thereof; and one oligonucleotide primer complementary to at least 6 nucleotides of *IS900* or a complement thereof.
- 10 19. A method as claimed in claim 12 step c) utilizes one oligonucleotide primer complementary to 10-12 contiguous nucleotides of SEQ ID NO. 1 or a complement thereof; and one oligonucleotide primer complementary to 10-12 nucleotides of *IS900* or a complement thereof.
- 15 20. A method as claimed in claim 12 wherein step c) utilizes one oligonucleotide primer complementary to substantially 15 contiguous nucleotides of SEQ ID NO. 1 or a complement thereof; and one oligonucleotide primer complementary to substantially 15 nucleotides of *IS900* or a complement thereof.
- 20 21. A method as claimed in claim 12 wherein step c) utilizes one oligonucleotide primer complementary to substantially 20 contiguous nucleotides of SEQ ID NO. 1 or a complement thereof; and one oligonucleotide primer complementary to substantially 20 nucleotides of *IS900* or a complement thereof.
22. A method as claimed in claim 12 wherein step c) of the method comprises identifying the presence of at least 6 contiguous nucleotides of the nucleic acid molecule comprising SEQ ID NO. 2 or a complement thereof.

23. A method as claimed in claim 12 wherein step d) of the method comprises identifying the presence of 10-12 contiguous nucleotides of the nucleic acid molecule comprising SEQ ID NO. 2 or a complement thereof.
24. A method as claimed in claim 12 wherein step d) of the method comprises identifying the presence of at least 15 contiguous nucleotides of the nucleic acid molecule comprising SEQ ID NO. 2 or a complement thereof.
25. A method as claimed in claim 12 wherein step d) of the method comprises identifying the presence of approximately 20 contiguous nucleotides of the nucleic acid molecule comprising SEQ ID NO. 2 or a complement thereof.
26. A method as claimed in claim 12 wherein step c) utilizes one oligonucleotide primer complementary to at least 6 contiguous nucleotides of SEQ ID NO. 2 or a complement thereof; and one oligonucleotide primer complementary to at least 6 nucleotides of IS900 or a complement thereof.
27. A method as claimed in claim 12 wherein step c) utilizes one oligonucleotide primer complementary to 10-12 contiguous nucleotides of SEQ ID NO. 2 or a complement thereof; and one oligonucleotide primer complementary to 10-12 nucleotides of IS900 or a complement thereof.
28. A method as claimed in claim 12 wherein step c) utilizes one oligonucleotide primer complementary to substantially 15 contiguous nucleotides of SEQ ID NO. 2 or a complement thereof; and one oligonucleotide primer complementary to substantially 15 nucleotides of IS900 or a complement thereof.
29. A method as claimed in claim 12 wherein step c) utilizes one oligonucleotide primer complementary to substantially 20 contiguous nucleotides of SEQ ID NO. 2 or a complement thereof; and one

oligonucleotide primer complementary to substantially 20 contiguous nucleotides of IS900 or a complement thereof.

30. The use of a probe comprising at least 6 contiguous nucleotides selected from the nucleic acid comprising SEQ ID NO. 2 or a complement thereof.
- 5 31. The use of a probe comprising substantially 10-12 contiguous nucleotides selected from the nucleic acid comprising SEQ ID NO. 2 or a complement thereof.
32. The use of a probe comprising at least 15 contiguous nucleotides selected from the nucleic acid comprising SEQ ID NO. 2 or a complement thereof.
- 10 33. The use of a probe comprising at least 20 contiguous nucleotides selected from the nucleic acid comprising SEQ ID NO. 2 or a complement thereof.
34. The use of SEQ ID NO.1 and/or SEQ ID NO. 2, or a fragment or complement thereof, to determine whether a strain of either a sheep type or a cattle type of *M. paratuberculosis* is present in a sample.
- 15 35. The use of SEQ ID NO.1, or a fragment or complement thereof, to distinguish any strain of *M. paratuberculosis* from any other strain of the MAI complex which may be present in a sample.
36. The use of SEQ ID NO.2, or a fragment or complement thereof, to distinguish any strain of *M. paratuberculosis* from any other strain of the MAI complex which may be present in a sample.
- 20 37. The use of SEQ ID NO.1, or a fragment or complement thereof, to distinguish any strain of *M. paratuberculosis* from any strain of the *M. tuberculosis* complex which may be present in a sample.

38. The use of SEQ ID NO.2, or a fragment or complement thereof, to distinguish any strain of *M. paratuberculosis* from any strain of the *M. tuberculosis* complex which may be present in a sample.
39. The use of SEQ ID NO. 1, or a fragment or complement thereof, to detect
5 the presence of *M. paratuberculosis* as a causative agent of Johne's disease or Crohn's disease.
40. The use of SEQ ID NO. 2, or a fragment or complement thereof, to detect the presence of *M. paratuberculosis* as a causative agent of Johne's disease or Crohn's disease.

FIGURE 1

SEQ ID NO. 1

```
1  GCTTGACAAC GTCATTGAGA ATCCCCTTGC TACTTAGCTG ACCTATCTAC
51 AGGCTGGGTC AGCATAATCG CAGCGTCGGA CAAGGCGTGG AGGAATCGCG
101 TCGCGACTTA TGCAACTCCT TCAAAACCGG CGTCGCGCCG CCGTGTGAAA
151 CGCAGGTCAA TCCGCACCGG GATGGTGATC GGTTCAAGTC GTCCGACCAT
201 GGGCGGTTGG GGACCCGGAC ACTCGAGACA TCACCCCTAG CTGATATCAG
251 CTTTAGCTGA TATCATGCGG TAGTGCGGAA ATGGGAGGTT GACCTGACTC
301 TCATCGAAGC CTGGATGGAT GCCCTCAATG ACGttgtcaa gc
```

FIGURE 2

SEQ ID NO. 2

TTGAAGTTGCCGAAGGTGCGAATCGAGCCGTCCGGTGTCTGAAGGCGACGGTGTCTGATCTTG
GTGTGCGGCAGGCGCAGGAACCTCGTCGATGAGGCCAGCTCGTCCATGATCCGCAGCGTG
GTCGGATGCACGGTGTCCGCCGGAAGTCGCGCAGGAAGTCGTTGTGCTTTTCCAAGACG
ACGACCGGGATTCCGGCCCGGGCAGCAGCAGCGCTGCACCATCCCGGCGGGACCGCCC
CCGGCAACGCACACCTGAGTCCGGATCAGTTGCGCAGGCTACCTCCCGGGTCCAACCACT
AGGCCGTCCAACCTCGACGGCCGCTACAGAGCTACACGAGCCCCGCCGAGAATCGAG
TCATGGAACCTCTATCGCTTCCGGCACCGCGCCCGGGGGATGTCTGAACGAGACGCTCCCA
TCCATACCGAGGCCAGGGTTGATGTCTTCCATCCAGGTGTTGTCTGAGCGAGGTCGTC
GCGTCAAACCTTCTTCCCGTCGATGATCAGTTTTTTGGTTGGAAGCAGAGAAGCTTTGGGAC
TTGTGCGCCGATGTTTCGTACGCGGAGCTTCACGACGAAAACTCGCCCTTGGCTTGCTGC
AAACTGAAGACCCCTTCTTAGTGGCCGAGCGATCGACACCAAGCACTTGAACTCGAAC
TTACCGTCTCGCACCGGGGACCCAGCGGGGGCGACGGCAGGCCCTACATGCTTTGGCTTC
GTCGGCGCGCTTGCCTTGGAGGGACCGACGCTGCCGTGTTTCGACTCCGCGACGGTCTTC
TTGTGCGCGTCGTTGCCGCGCTGGCGATGGCAATCAGGACCACCACTACGGCTCCGGCG
CCTAGGATCCACTTCTTATCGGACTTCTTCTGGCGGCTGGGCTTCACGGGCTGGCCAACG
CCGGGTGGCGGCGCGGGAAATGGCGCATAACTTTCGGTCCACGCTGTGCCGTCTGAAGTAC
CGCTGGCGGCTCGGATTAGATGGATCGGGATACCAACCCGCTGTTGGCTGCGTCATGAAG
TCCCCCTTGCTACTTAGCTGACCTATCTACAGGCTGGGTGAGCATAACCGCAGCGTCGGAC
AAGGCGTGGAGGAATCGCGTCGCGACTTATGCAACTCCTTCAAAACCGGCGTCGCGCCGC
CGTGTGAAACGCAGGTCAATCCGCACCGGGATGGTGATCGGTTCAAGTCGTCCGACCATG
GGCGGTTGGGGACCCGGACACTCGAGACATCACCCCTAGCTGATATCATGCGGTAGTGCG
GAAATGGGAGGTTGACCTGACTCTCATCGAAGCCTGGATGGATGCCCTCGATGACGAGGA
GTACGACAACCTTGATAGCAGCGCTCGAGCAGTTGGAGGAACACGGTCCAATTACCCGGCG
GCCGTTTGTGGACACCCTTGAAGGGTCAAGGCACCCGAACATGAAGGAGCTCCGTCCGCG
CCCCACGAAAGCTGGAGCCACATTTCGCGTGCTATTTCGCTTCGATACCCGCTCGCGGGC
GATCATGTTGATCGCTGGAGACAAGGCGGGCAATTGGTCCAAGTGGTATGCCAAGCACAT
TCCCATTGCCGACGAGCTCTTCGATGCTCACCAAAAGCGTTTACACAAGGCAGCAGCCAA
AGCCACCAATCGGAAACCAAGGAAGGGAAAGAAACGATGACCAATCTTGATGACATGCGC
CGTCGCCGTCCCGGCAACCGGACCCGTATCGACGCGATCAAGGCGGAGATGGACCGCGAG
GTTGCCAGTACCGTCTCCGCGAACTGCGCGAGGCTGCCGGCTACACGCAGACGACTCTC
GCTGCGGCTATTGGCGTCGGGCAGAACCGCGTCTCGCAGATGGAACATGGCGATCTGGGC
ACGAGCCGGGTGACACTCTCCGCAAGTACGTGGAAGCGACTGGGGGTGAGCTGGAAGTG
TCCGTCAAGCGTCCGGACGGGTACGGGTTCTCCTCAGCCTGTAAAGCGCGACGACCGAG
ACCCAGGGCGAAGGCTTCGAGAACGGTGTCCAGGTACCGGCTGCGGCTTGTGATTCTCGG
GTGCCCTCGGCGCTCAGAAGCAGTCGAATGACGGGGCCATCGATACAGAATCGACGGAGA
ACCGAAGCAGTACAACAGATCTCGTCGCACATCGGCCTCAACTCGATCGCGGCACAGATC
AGGTAACCGTATTTCGCGGACGTGCGCAAGACCGACTCCGATGCAACACGATCACCCGTCT
AGCACGGCTAGAGTCACCTGTGCGACTGTGCGCGACCGCTGTCTCCACCGAAGACGAC
GAGGACACCACGGCGAAGTACCACCC

FIGURE 3

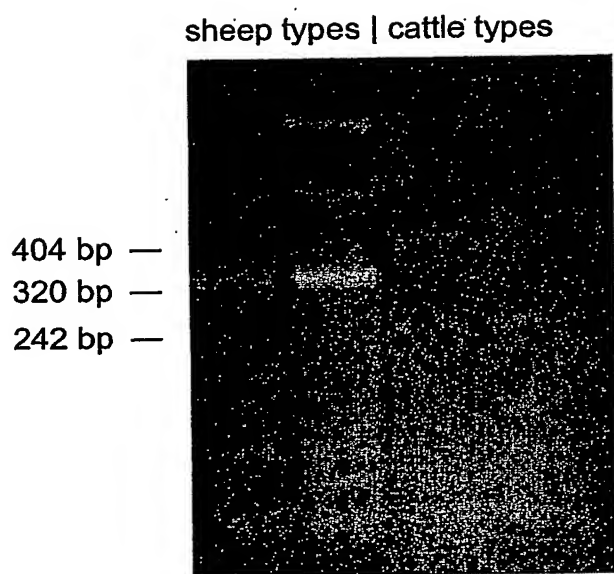


FIGURE 4

cattle 850 cacttcttatcggacttcttctggtggcggtgggcttcacgggctggccaacgccgggtggcgggcgcgggaa
DMC531

cattle 920 atggcgcatataactttcgggtccacgctgtgcccgtcgaagtaccgctggcggtcggattagatggatcggg

cattle 990 ataccaaccgctgttggctgcgtcatGAAGTCCCCTTGCTACTTAGCTGACCTATCTACAGGCTGGGTC
sheep 1GCTTGACAACGTCATTGAGAAATCCCCTTGCTACTTAGCTGACCTATCTACAGGCTGGGTC
DMC529
DMC136

cattle 1060 AGCATAACCGCAGCGTCGGACAAGGCGTGGAGGAATCGCGTCGCGACTTATGCAACTCCTTCAAACCGG
sheep 61 AGCATAATCGCAGCGTCGGACAAGGCGTGGAGGAATCGCGTCGCGACTTATGCAACTCCTTCAAACCGG

cattle 1130 CGTCGCGCCGCCGTGTGAAACGCAGGTCAATCCGCACCGGGATGGTGATCGGTTCAAGTCGTCCGACCAT
sheep 131 CGTCGCGCCGCCGTGTGAAACGCAGGTCAATCCGCACCGGGATGGTGATCGGTTCAAGTCGTCCGACCAT
DMC533

cattle 1200 GGGCGGTTGGGGACCCGGACACTCGAGACATCACCCC.....TAGCTGATATCATGCGG
sheep 201 GGGCGGTTGGGGACCCGGACACTCGAGACATCACCCC TAGCTGATATCAGCTT TAGCTGATATCATGCGG

cattle 1254 TAGTGCGGAAATGGGAGGTTGACCTGACTCTCATCGAAGCCTGGATGGATGCCCTCCATGACGAGGAGTA
sheep 271 TAGTGCGGAAATGGGAGGTTGACCTGACTCTCATCGAAGCCTGGATGGATGCCCTCAATGACGttgtcaa

DMC505

cattle 1324 CGACAACTTGATAGC
sheep 341 gc.....

3' end of DMC136

FIGURE 5

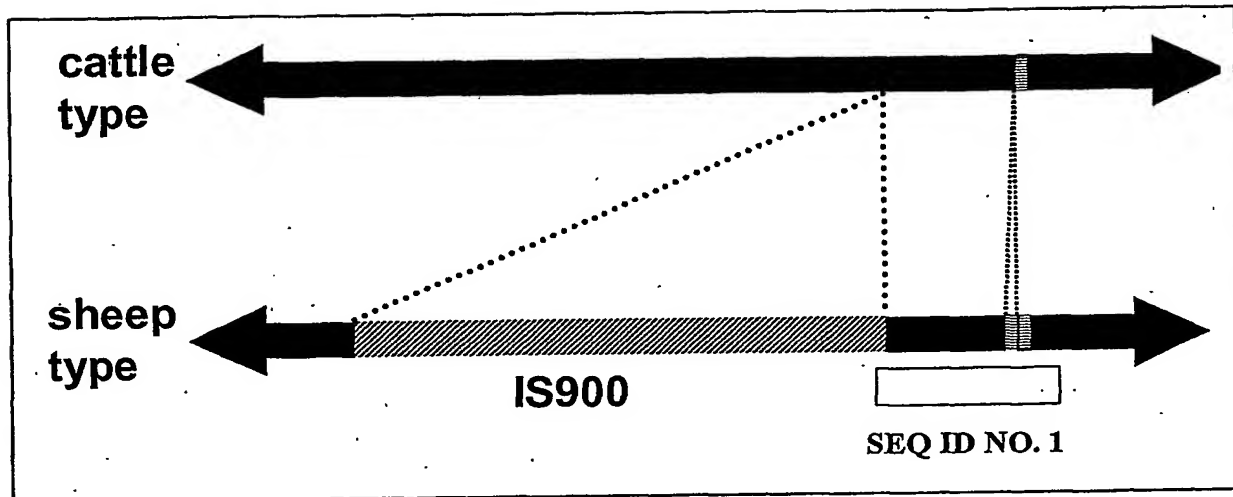


FIGURE 6

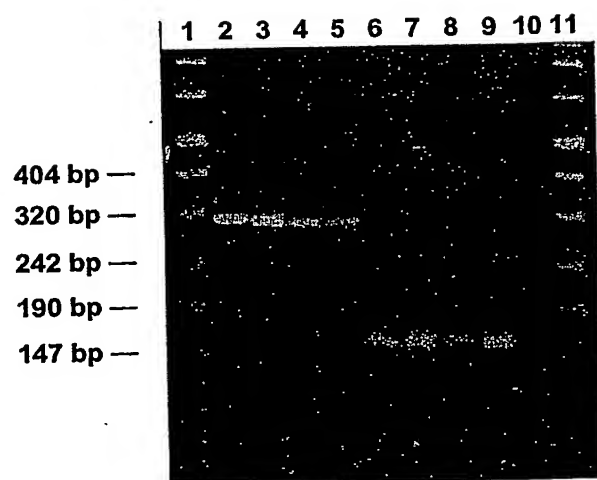
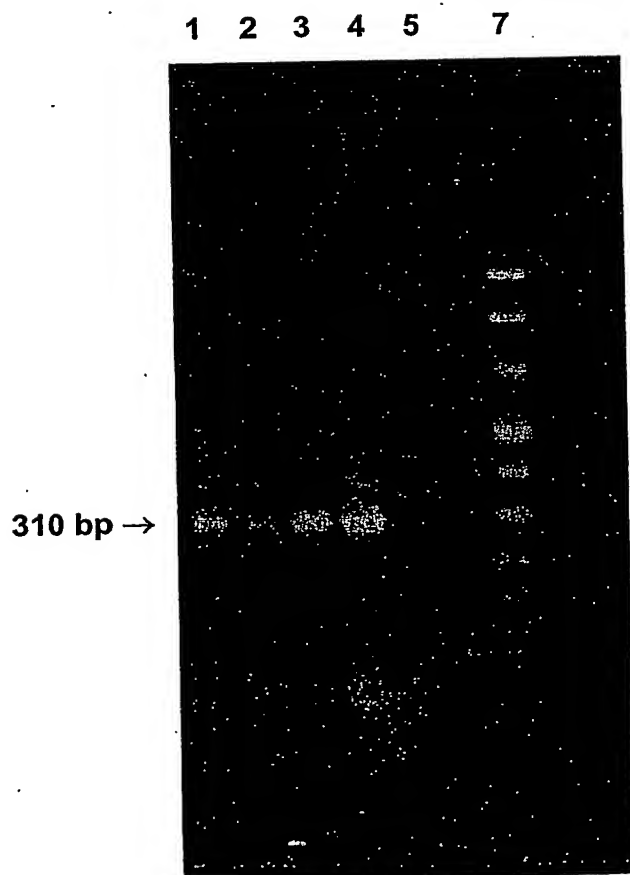


FIGURE 7



INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ03/00119

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. ⁷: C12Q 1/68 C12N 15/11 C12N 15/31

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

SEE ELECTRONIC DATABASES

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SEE ELECTRONIC DATABASES

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DGENE (Seq ID No 1 and 2); Pubmed, USPTO internet search (mycobacterium, paratuberculosis, sheep, cattle)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P/X	EP1223225 A (STICHTING NEDERLANDS INSTITUUT VOOR ZUIVELONDERZOEK) 17 July 2002. (See description, also Seq ID No 3)	3-5, 12, 13, 9, 14-21, 34, 35, 37, 39
X	Coetsier, C. <i>et al.</i> 2000. Duplex PCR for differential identification of <i>Mycobacterium bovis</i> , <i>M. avium</i> , and <i>M. avium</i> subsp. <i>Paratuberculosis</i> in formalin-fixed paraffin-embedded tissues from cattle. <i>Journal of Clinical Microbiology</i> . 38(8):3048-3054. (See whole document)	12, 13
X	Whittington, R.J. <i>et al.</i> 2000. Molecular epidemiology of <i>Mycobacterium avium</i> subsp. <i>Paratuberculosis</i> : IS900 restriction fragment length polymorphism and IS1311 polymorphism analyses of isolates from animals and a human in Australia. <i>Journal of Clinical Microbiology</i> . 38(9):3240-3248. (See whole document)	12, 13

☒ Further documents are listed in the continuation of Box C☒ See patent family annex

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search
7 August 2003

Date of mailing of the international search report 18 AUG 2003

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/NZ03/00119

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>Bull, T.J. <i>et al.</i> 2000. Characterization of IS900 loci in <i>Mycobacterium avium</i> subsp. <i>Paratuberculosis</i> and development of multiplex PCR typing. Microbiology. 146:2185-2197. (See whole document)</p> <p>And, GenBank database accession No. AJ250016. Bull, T.J <i>et al.</i> <i>Mycobacterium avium</i> subsp. <i>Paratuberculosis</i> insertion sequence IS900, Locus 2. 26 September 2000.</p> <p>And, GenBank database accession No. AJ011838. Bull, J. <i>et al.</i> <i>Mycobacterium avium paratuberculosis</i> <i>alkA</i> (partial), <i>hed</i>, <i>p43</i> and <i>ogt</i>. 26 September 2000.</p>	3-5, 9, 12-21, 34, 35, 37, 39
X	<p>US5968815 A (Murray <i>et al</i>) 19 October 1999. (See Seq ID No 8)</p>	3-5
X	<p>Bauerfeind, R. <i>et al.</i> 1996. Molecular characterization of <i>Mycobacterium paratuberculosis</i> isolates from sheep, goats, and cattle by hybridization with a DNA probe to insertion element IS900. Journal of Clinical Microbiology. 34(7):1617-1621. (See whole document)</p>	12, 13
X	<p>EP0288306 B (BIOSCIENCE INTERNATIONAL INC) 13 December 1995. (See description, also Figure 4)</p>	3-5
X	<p>AU632383 B (MASSEY UNIVERSITY) 23 May 1991. (See description, also Fig 2)</p>	3-5
T	<p>Collins <i>et al.</i> 2002. <i>Mycobacterium avium</i> subsp. <i>Paratuberculosis</i> strains from cattle and sheep can be distinguished by a PCR test based on a novel DNA sequence difference. Journal of Clinical Microbiology. 40(12):4760-4762. See whole document.</p>	1-40

INTERNATIONAL SEARCH REPORT

PCT/NZ03/00119

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report			Patent Family Member				
EP	288306	AU	16286/88	CA	1340172	DK	5242/89
		EP	356450	NO	885756	NZ	224351
		US	5225324	WO	8808456		
AU	632383	NZ	231429				
US	5968815	EP	666917	FR	2682967	NZ	244901
		WO	9308284				
EP	1223225	NONE					
END OF ANNEX							

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